Chapter 1

Introduction:
Structural features of the glutamate transporter family

Published in *Microbiol. Mol. Biol. Rev*. **63** (2): 293-307 (1999)

Summary

Neuronal and glial glutamate transporters remove the excitatory neurotransmitter glutamate from the synaptic cleft and thus prevent neurotoxicity. The proteins belong to a large and widespread family of secondary transporters, including bacterial glutamate, serine and C4-dicarboxylate transporters, mammalian neutral amino acid transporters and an increasing number of bacterial, archaeal and eukaryotic proteins that have not yet been functionally characterized. More than hundred members of the glutamate transporter family were found in the databases on the basis of sequence homology. The amino acid sequences of the carriers have diverged enormously. Homology between the members of the family is most apparent in a stretch of approximately 150 residues in the C-terminal part of the proteins. This region contains four reasonably well-conserved sequence motifs, all of which have been suggested to be part of the translocation pore or substrate binding site. Phylogenetic analysis of the C-terminal stretch revealed the presence of five subfamilies with characterized members, (i) the eukaryotic glutamate transporters, (ii) the bacterial glutamate transporters, (iii) the eukaryotic neutral amino acid transporters, (iv) the bacterial C4-dicarboxylate transporters and (v) the bacterial serine transporters. A number of other subfamilies that do not contain characterized members have been defined. In contrast to their amino acid sequences the hydropathy profiles of the members of the family are extremely well conserved. Analysis of the hydropathy profiles has suggested that the glutamate transporters have a global structure that is unique among secondary transporters. Experimentally, the unique structure of the transporters was recently confirmed by membrane topology studies. Although there is still controversy about part of the topology, the most likely model predicts the presence of eight membrane-spanning α-helices and two pore-loop structures which are unique among secondary transporters but which may resemble pore-loops found in ion channels. A second distinctive structural feature is the presence of a highly amphipathic membrane-spanning helix that provides a hydrophilic path through the membrane. Recent data from analysis of site-directed mutants and studies on the mechanism and pharmacology of the transporters are discussed in relation to the structural model.

I. Introduction

Eukaryotic, bacterial, and archaeal cells contain a large number of integral membrane proteins and protein complexes involved in solute transport across the membrane (85). In *Escherichia coli* as many as 10.8% of all chromosomal genes code for membrane transport proteins (118). All known and putative transport proteins have recently been classified in different families based on their sequence similarities. This led to the identification of 174 transporter families, of which secondary transporters represent the largest functional category, comprising 77 families (137).

Secondary transporters use the free energy stored in ion and/or solute gradients over the membrane to drive transport. Since three-dimensional structures of secondary transporters have not yet been solved, models for their structures are based on biochemical data and on (computational) analysis of their amino acid sequences. A typical secondary transporter consists of a single polypeptide component that forms a bundle of membrane-spanning α-helices connected by loops of various sizes. Many of these proteins contain 12 membrane-spanning segments, but different numbers are not exceptional (137). The 77 families of secondary transporters do not represent as many different global structures. It has been shown by hydropathy profile analysis that many of the families belong to the same structural class (chapter 2) (94,95). One of the families that forms a separate structural class is the glutamate transporter family, also called dicarboxylate/cation symporters (DAACS; transporter classification 2.23) (137).

The glutamate transporter family includes transporters that are found in neurons and glial cells in the mammalian central nervous system and catalyze the reuptake of the excitatory neurotransmitter glutamate from the synaptic cleft (40,71,121,152). Excessive amounts of extracellular glutamate, associated with several diseases, cause neuronal destruction via activation of the N-methyl-D-aspartate receptors (103,112). The neuronal and glial glutamate transporters are believed to prevent excitotoxicity of glutamate (92,133,155) and may help to end the excitatory signal together with diffusion (104,115). Since these proteins have been implicated in numerous neurological disorders, they have attracted much attention and have been well characterized.

The number of known transporters in the glutamate transporter family has grown rapidly during the last years, and many members of the family have now been characterized, including retinal glutamate transporters from vertebrates (7,35), mammalian neutral-amino-acid transporters (9,91,142), and bacterial nutrient uptake proteins (36,158,159). Because of the variety of transporters that are being studied in the glutamate transporter family and the numerous techniques used to study them, the structural features of the transporter family are now rapidly being unraveled. This review focuses on the diversity of transporters in the family and on their structural properties. Physiological and pathological aspects have recently been reviewed elsewhere (70,172).

II. The family

1. Substrate specificity

The members of the glutamate transporter family that have been functionally characterized can be classified in three groups based on their substrate specificity (Table 1): C4-dicarboxylate transporters (found in bacteria), glutamate/aspartate transporters (found in bacteria and eukaryotes) and neutral-amino-acid transporters (found in bacteria and eukaryotes). The bacterial C4-dicarboxylate carriers transport the tricarboxylic acid cycle intermediates succinate, fumarate, and malate (43,44). In addition, the transporter from

Rhizobium meliloti uses aspartate (185) and the transporters from *Escherichia coli* and *Salmonella typhimurium* use orotate as substrate (10). It is not known which transmembrane ion or solute gradient(s) provides the free energy to drive transport.

Table 1. Substrate specificity of the members of the glutamate transporter family

Subfamily	King-dom[a]	Substrates[b]	Mode of energy coupling
C4-dicarboxylate transporters	B	Succinate, fumarate, malate, (orotate, aspartate)	unknown
Glutamate/aspartate transporters	B, E	Glutamate, aspartate, glutamate analogues	H^+ symport; H^+/Na^+ symport; H^+/Na^+ symport-K^+ antiport exchange; Na^+ symport
Neutral amino acid transporters	B, E	Alanine, serine, cysteine, threonine (asparagine, glutamine)	

[a] B, Bacteria; E, Eukarya.
[b] High affinity substrates are shown; substrates between parentheses are not accepted by all members of the subfamilies

All glutamate transporters use L-glutamate and L-aspartate as high-affinity substrates (K_m [Glu] < 100µM). In addition, several (nonphysiological) glutamate analogues are transported (71,121,152,158,161,183). The bacterial proteins catalyze the electrogenic symport of glutamate with at least two cations (158,160,161). The GltP proteins from *E. coli* and *Bacillus subtilis* use protons to drive the uptake of glutamate, whereas the GltT proteins from *Bacillus stearothermophilus* and *Bacillus caldotenax* can replace one proton with a sodium ion (57). Na^+ is preferentially used at elevated temperatures, but the selectivity for sodium ions is lost when these transporters are expressed in *E. coli* (160). These observations indicate that minor conformational changes may alter the cation specificity.

The eukaryotic glutamate transporters catalyze the electrogenic symport of glutamate with two or three sodium ions and one proton, whereas one potassium ion is antiported (5,11,37,77,195). The exact number of transported sodium ions is still a matter of debate (70,72,90,195), but a stoichiometry of three sodium ions per glutamate is generally favored (90,195). Transport of glutamate by the eukaryotic proteins is strictly dependent on sodium ions, but not all Na^+ binding sites are equally selective. For the glutamate transporter EAAT2 of rats (also known as GLT-1), it was shown that only one Na^+ binding site strictly requires sodium ions whereas lithium ions can replace Na^+ in the others (51). Cesium, rubidium, and ammonium ions can replace K^+ to various extents (79).

The high-affinity substrates for the mammalian neutral-amino-acid transporters are alanine, serine, cysteine, and threonine (K_m < 100 µM), but some members (ACST2 from mice, humans, and rabbits) show a broader substrate specificity and accept glutamine and asparagine with high affinity and several other amino acids, including glutamate, with lower affinity (K_m > 300 µM) (9,80,81,154,168). The human neutral amino acid transporter ASCT1 is an obligate exchanger that does not mediate a net flux of amino acids (194). Exchange of the amino acids is electroneutral and accompanied by a symmetrical exchange of one Na^+. Mouse ASCT2 probably uses the same transport mode (168). Recently, a serine transporter from *E. coli* that is a Na^+ symporter (114) was characterized.

Both the eukaryotic glutamate transporters and the eukaryotic neutral-amino-acid carriers mediate thermodynamically uncoupled ion fluxes. Besides substrate-independent cation leaks, substrate-dependent Cl^- currents have been reported (7,15,35,40,72,120,149,173,180,194). The extent of the substrate-dependent Cl^- flux differs for the various members of the family but is very high in the glutamate transporters EAAT4 and EAAT5 from the human brain and retina and EAAT5A from the salamander retina (7,35,40). Apparently, the proteins have a dual function as glutamate transporters and glutamate-gated chloride channels. Glutamate transporters with chloride channel activity are the first examples of glutamate-gated chloride channels in vertebrates. Glutamate-gated chloride channels were found before in

invertebrates, which use glutamate as an inhibitory neurotransmitter. The invertebrate glutamate-gated chloride channels are not related to the glutamate transporters but show similarity to acetylcholine-, serotonine-, GABA- and glycine-gated channels (25,177).

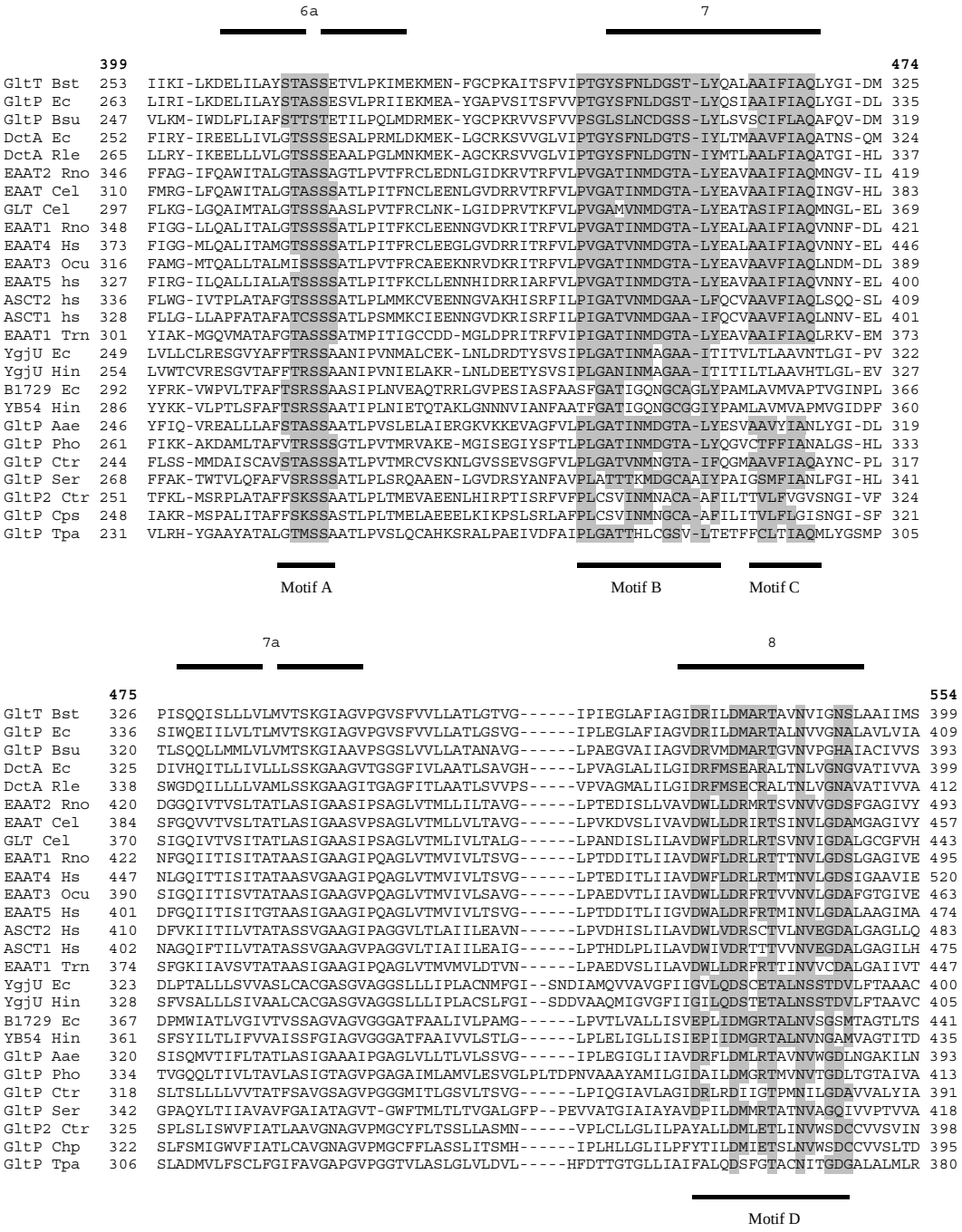

Figure 1. Multiple sequence alignment of a stretch of approximately 150 residues near the C-terminus of the transporters. The alignment was made with the program CLUSTALX (156). A representative set of 26 members of the glutamate transporter family is shown. Bold numbers refer to the positions in the multiple sequence alignment and correspond to the numbers in Fig. 4. Normal numbers refer to the residue numbers of the individual sequences. Bars below and above the sequences indicate the positions of the conserved motifs (motif A-D, highlighted) and the positions of the transmembrane segments, respectively. Abbreviated organism names: Bst, *Bacillus stearothermophilus*; Ec, *Escherichia coli*; Bsu, *Bacillus subtilis*; Rle, *Rhizobium leguminosarum*; Rno, *Rattus norvegicus*; Cel, *Caenorhabditis elegans*; Hs, *Homo sapiens*; Ocu, *Oryctolagus cuniculus*; Trn Hin, *Haemophilus influenzae*; Aae, *Aquifex aeolicus*; Pho, *Pyrococcus horikoshii*; Ctr, *Chlamydia trachomatis*; Ser, *Saccharopolyspora erythraea*; Cps, *Chlamydia psittaci*; Tpa, *Treponema pallidum*.

2. Diversity and phylogeny

More than one hundred protein sequences from the *Eucarya*, *Bacteria*, and *Archaea*, belonging to the glutamate transporter family, were found in the protein and translated nucleotide databases by using the BLAST facility (4). The sequences vary in length from 396 to 581 residues, with the bacterial ones being significantly shorter (396 to 491 residues) than the eukaryotic ones (479 to 581 residues). Several organisms contain multiple paralogues of the family. The bacteria *E. coli* and *B. subtilis* have four paralogues each, whereas in eukaryotes up to nine (in *Homo sapiens*) have been found so far. A multiple-sequence alignment was made with the CLUSTALX program (156). A stretch of about 150 residues in the C-terminal part of the sequences is better conserved than the N-terminal part (Fig. 1). This C-terminal part of the alignment, which is unambiguous and contains very few gaps, was used to construct the phylogenetic tree shown in Fig. 2. The tree includes a subset of 35 members of the family that do not contain pairs of sequences with more than 70% identical residues. Since more similar pairs cluster closely together in the tree, they are less informative. Several distinct subfamilies can be recognized in the phylogenetic tree of the glutamate transporter family. All eukaryotic members cluster in one branch of the tree. They have at least 31% identical residues over the full length. The neutral amino acid transporters and the glutamate transporters clearly form separate groups within the eukaryotic branch, supporting the notion that substrate specificity correlates with phylogenetic classification (117). However, the eukaryotic and bacterial glutamate transporters that have similar substrate specificity do not cluster in the tree but belong to separate subfamilies. Likewise, the eukaryotic neutral-amino-acid transporters and the bacterial serine transporters are found in different branches of the tree. The analysis suggests that phylogenetic clustering of members of the family is determined hierarchically, first by the evolutionary position of the host organism and second by the substrate specificity (136).

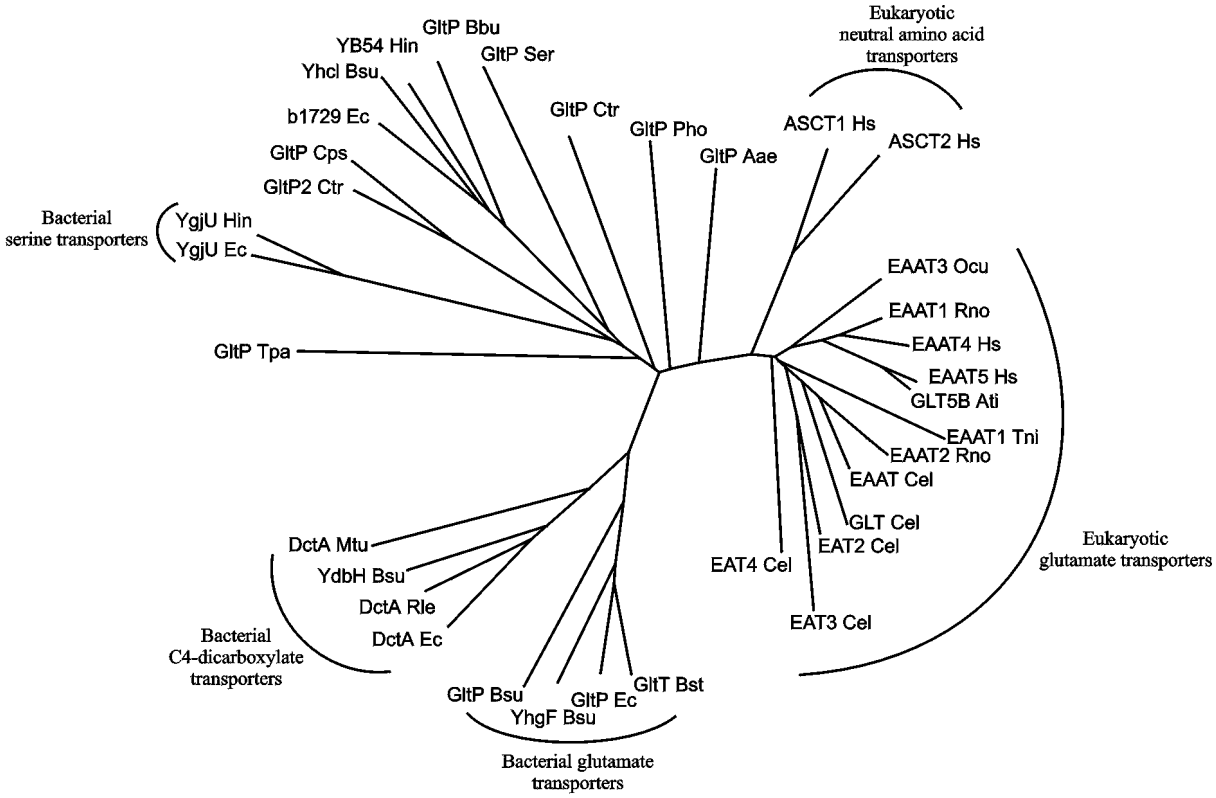

Figure 2. Phylogenetic tree of 35 members of the glutamate transporter family. The set of 35 members does not contain pairs of sequences with more than 70% identical residues. The tree is based on the part of the multiple sequence alignment shown in Fig. 1. It was drawn using the DRAWTREE program from the PHYLIP package (41). Abbreviated transporter names were taken from Fig. 1 and: Bbu, *Borrelia burgdorferi*; Ati, *Ambystoma tigrinum*; Tni, *Trichoplusia ni*; Mtu, *Mycobacterium tuberculosis*.

The bacterial glutamate transporters are found in the same major branch as the bacterial C_4-dicarboxylate transporters, but within this branch they form separate clusters. The bacterial glutamate transporters have more than 44% identical residues; the C_4-dicarboxylate transporters share at least 40% identical residues. The percentage of identical residues between the members of the GltP and DctA subfamilies varies from 27 to 42%. In both groups, a protein from *B. subtilis* that has not been functionally characterized is present (YhfG and YdbH, respectively). It is likely that these proteins have the same substrate specificity as the other members of the subfamilies to which they belong. The two bacterial subfamilies are more closely related to each other than to the eukaryotic subfamily. The bacterial and eukaryotic members have 18 to 28% identical residues.

The serine transporter YgjU of *E. coli* is only distantly related to the other characterized members of the family (with at most 20% identical residues). It is likely that the related protein YgjU of *Haemophilus influenzae* (56% identity) is also a serine transporter. The bacterial serine transporters are found in a cluster of proteins that branches off from a point near the center of the tree, indicating that they have diverged from the other sequences early in evolution (117). The cluster contains a large number of uncharacterized proteins that are only distantly related to the characterized members of the glutamate transporter family. The uncharacterized proteins are found not only in organisms like the chlamydiae (187), which are not closely related to the organisms in the clusters with characterized proteins, but also in organisms like *E. coli* and *B. subtilis*, which have well-characterized members. Therefore, it is likely that at least some of the uncharacterized members of the family are proteins with different substrate specificity from the transporters that have been characterized so far. Among the uncharacterized members, several subfamilies can be distinguished that are likely to consist of proteins with similar, currently unknown substrate specificity. Hence, b1729 of *E. coli*, YB54 of *H. influenzae*, GltP of *Borrelia burgdorferi*, and YhcL of *B. subtilis* probably are orthologues with the same function. The same applies to GltP2 of *Chlamydia trachomatis* and GltP of *Chlamydia psittaci*. Two other proteins, GltP2 of *Treponema pallidum* and GltP2 of *B. burgdorferi*, are not included in the phylogenetic tree (Fig. 2) because they have diverged so far that it is difficult to unambiguously align them (they have at most 19 and 17% of residues identical to those of other members of the family, respectively). Nevertheless, they are likely to belong to the family because of the (partial) presence of conserved motifs and characteristic features of the hydropathy profiles (see below).

Finally, two sequences occupy separate positions in the tree. The glutamate transporter homologue GltP Aae from the hyperthermophilic bacterium *Aquifex aeolicus* is more closely related to the eukaryotic transporters (32 to 39% identical residues) than to the bacterial members (22 to 32% identical residues). The separate position of GltP of *A. aeolicus* in the tree reflects the unique evolutionary position of this organism (27). The only archaeal sequence in the tree (GltP Pho) is equally closely related to the bacterial and eukaryotic sequences (approximately 30% of identical residues) and branches from a point near the center of the tree, reflecting the distinct phylogenetic position of the archaea (188).

III. Structural analysis

1. Hydropathy profile analysis

Although the amino acid sequences of the members of the glutamate transporter family have diverged considerably, their hydropathy profiles are remarkably similar (chapters 2 and 4) (73,94,147) (Fig. 3). It is a general property of families of integral membrane proteins that the hydropathy profiles of the members are better conserved than the primary structures. The conservation of hydropathy profiles is a reflection of the conservation of the global structure

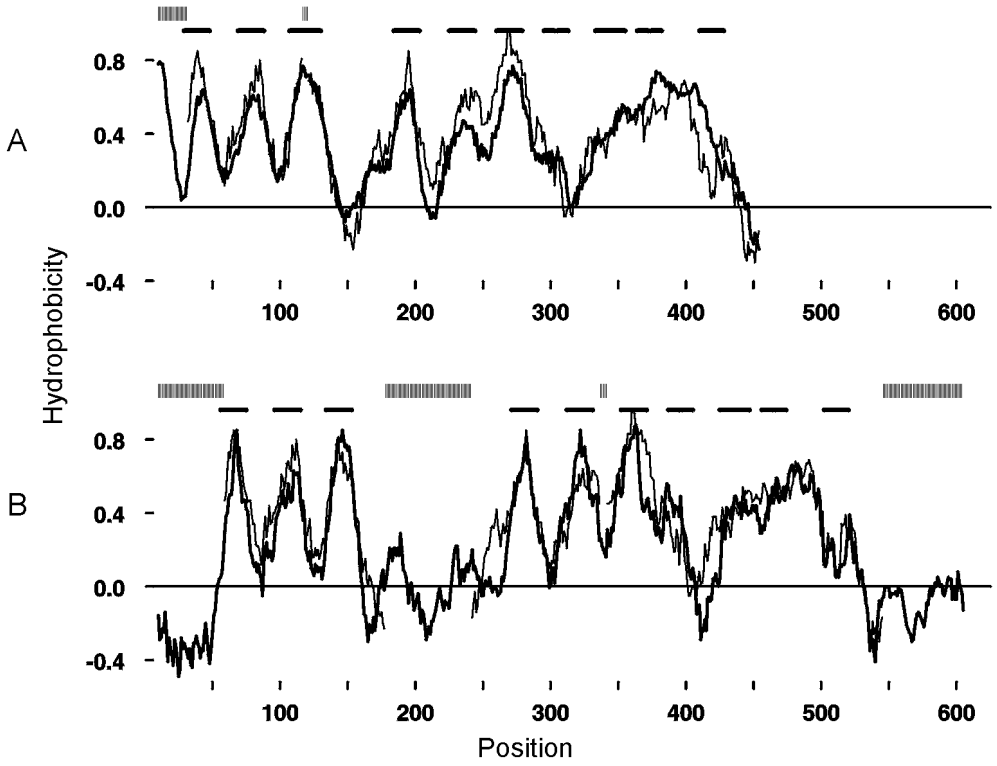

Figure 3. Alignment of average hydropathy profiles. Alignment of the profiles of (A) the subfamily of bacterial glutamate transporters (thin line) and the subfamily consisting of b1729 of *E. coli*, YB54 of *H. influenzae*, GltP of *B. burgdorferi* and YhcL of *B. subtilis* (bold line) and (B) the subfamilies of bacterial and eukaryotic glutamate transporters (thin and bold lines, respectively). The profiles were aligned according to Lolkema and Slotboom (chapter 2) (94) using a window of 19 amino acids. Vertical and horizontal bars above the profiles indicate the positions of gaps in the sequences and the positions of the transmembrane segments, respectively. The profiles are almost superimposable even though the sequences have considerably diverged. The bacterial glutamate transporters and the subfamily containing YB54 of *H. influenzae* share 18-24% identical residues (A) whereas the subfamilies of bacterial and eukaryotic glutamate transporters have 22-29% identical residues (B). The subfamily containing YB54 of *H. influenzae* has an extra hydrophobic segment at the N-terminus.

of the members of a family (chapter 2) (94,95). Quantitative comparison of the hydropathy profiles of different families of secondary transporters has recently been used to group the transporters into four major structural classes. It was shown that the hydropathy profile of the glutamate transporter family is likely to reflect a global structure that is unique among secondary transporters (chapter 2) (94).

The average hydropathy profile derived from the multiple sequence alignment of a subset of 35 members of the family (Fig. 4A) emphasizes the conserved characteristics of the profiles, which include six alternating hydrophobic and hydrophilic regions in the N-terminal half and a large hydrophobic region in the C-terminal half. The subset of 35 members does not contain any pairs of sequences with more than 70% identical residues, so that the average profile is not biased toward the profile of a particular subfamily. The eukaryotic and prokaryotic members differ predominantly in the length of their hydrophilic regions. Three hydrophilic stretches are considerably longer in the eukaryotic proteins: the N-terminal and C-terminal extensions and the region between the third and fourth hydrophobic peaks, which is glycosylated in the eukaryotic members (23). As is commonly observed for families of integral membrane proteins, the gaps in the multiple-sequence alignment are found almost exclusively in the hydrophilic stretches (Fig.4A).

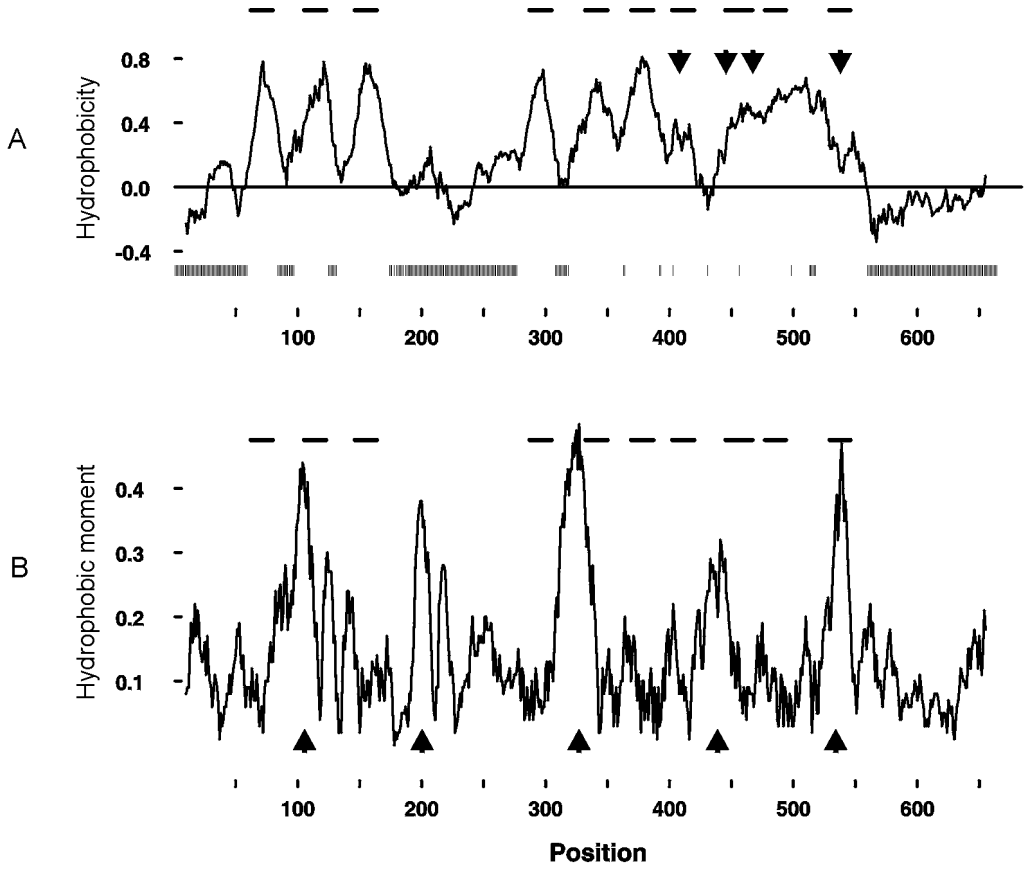

Figure 4. Average profiles of hydropathy (A) and hydrophobic moment (B) of the glutamate transporter family. The set of 35 members (see Fig. 2) which does not contain pairs of sequences with more than 70% identical residues was used. Vertical and horizontal bars above the profiles indicate the positions of gaps in the sequences and the positions of the transmembrane segments, respectively. Position numbers refer to positions in the multiple sequence alignment and correspond to the bold numbers in Fig. 1. In (A) a window of 19 residues was used and the arrows point to the positions of the conserved motives A-D. In (B) a window of 21 residues and a period of 3.6 residues, appropriate for α-helical structures, was used and the arrows point to the five conserved putative amphipathic helices (AH1-5 from left to right).

2. Membrane topology

The six hydrophobic segments in the N-terminal part of the glutamate transporters were predicted to be transmembrane helices in several reports (71,121,152,161). Experimentally, the membrane topology of rat EAAT1 (GLAST) was determined by monitoring the glycosylation state of C-terminally truncated transporters fused to a reporter peptide with glycosylation sites (182) and the topology of rat EAAT2 (GLT-1) was assessed by labeling of single-cysteine mutants (50). Both studies confirmed the presence of six membrane-spanning segments in the N-terminal half of the transporters. Also, the results of trypsin digestion experiments on rat EAAT2 in membrane vesicles are consistent with the presence of six N-terminal membrane helices. These studies showed that the N terminus of the protein is located at the cytoplasmic side of the membrane whereas the large hydrophilic region between the third and fourth hydrophobic segments is extracellular (51), which is in agreement with the observed glycosylation of this loop in the eukaryotic transporters (23).

The C-terminal half of the proteins does not contain clear alternating regions of high and low hydrophobicity. Therefore, it is difficult to predict the position of transmembrane segments from the hydropathy profile (67,71,121,152,161). Although the membrane topology of the C-terminal halves of several members of the glutamate transporter family (rat EAAT1 and

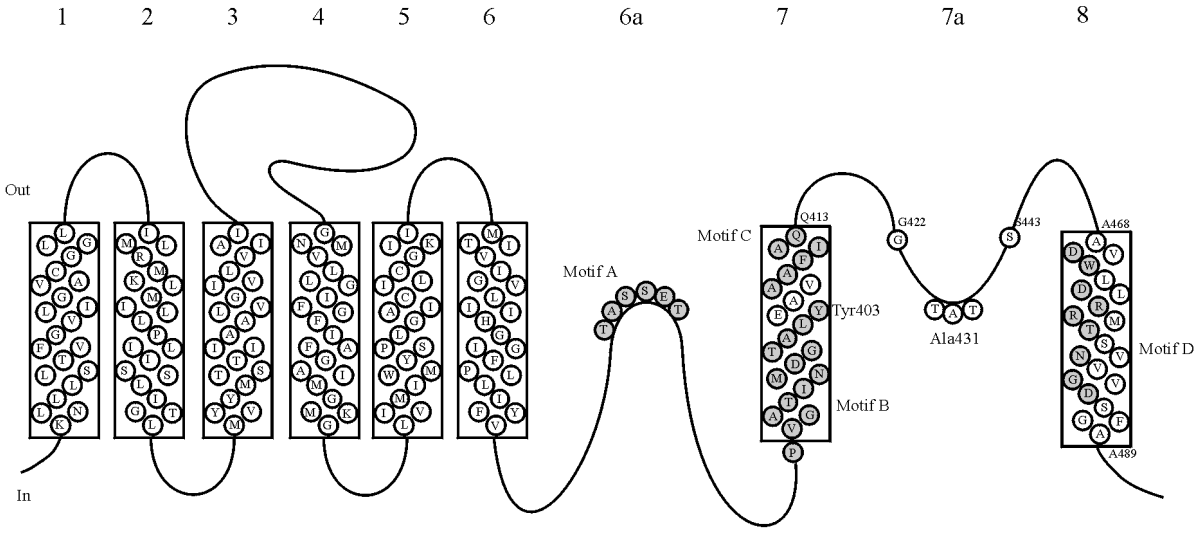

Figure 5. Model for the membrane topology of the glutamate transporters. The sequence of EAAT2 (GLT-1) from rat is shown. Conserved motifs A, B and C and the conserved hydrophilic face of helix 8 (motif D) are indicated. Tyrosine 403, which is involved in potassium binding and is accessible from either side of the membrane depending on the presence of substrates is located in the middle of helix 7.

EAAT2, *B. stearothermophilus* GltT, and human EAAT1) has been assessed with a variety of experimental techniques (chapters 4, 5 and 6) (50-52,139,141,147,148,182), it remains somewhat controversial. In the most generally accepted model the six N-terminal α-helices are followed by a reentrant loop entering the membrane from the cytoplasmic side, a seventh membrane-spanning helix, a reentrant loop entering the membrane from the extracellular side and, finally, an eighth membrane-spanning segment, leaving the C-terminus in the cytoplasm (Fig. 5).

The controversy about the membrane topology is confined to three regions in the proteins. The area containing the first reentrant loop has been studied in three transporters (rat EAAT2 (52), *B. stearothermophilus* GltT (chapter 5 (148)) and human EAAT1 (139)) using cysteine-scanning mutagenesis and two different models were proposed. However, as discussed in chapter 7, all results are likely to be consistent with the consensus model presented in Fig. 5. The second reentrant loop was found by cysteine-scanning in rat EAAT2 but not in human EAAT1. In the latter transporter the region between membrane-spanning segments 7 and 8 was predicted to be extracellular. However, the hydrophobic nature of the region as well as studies on the membrane topology of GltT using C-terminally truncated versions of the protein coupled to the reporter protein alkaline phosphatase (chapter 4 (147)), suggest that the region is membrane embedded. Finally, the position and secondary structure of membrane-spanning segment 8 has been a matter of debate. In chapter 6 an experimental study is described that confirms predictions that segment 8 is α-helical and highly amphipathic (146,147).

Clearly, the structure of the C-terminal part of the transporters is unusual containing reentrant loops or pore-loop structures, which were previously found in ion channels only (32,46,99,100,107). A model for the membrane topology of the C-terminal half of the transporters is shown in Fig. 5. The positions of the membrane-spanning segments are indicated in Fig. 1 and 4a.

3. Periodicity properties

The members of the glutamate transporter family were examined for patterns of residue hydrophobicity and residue conservation (or substitution) in their amino acid sequences

(chapter 4) (34,34,147). Fig. 4b shows the average periodicity profile of amino acid hydrophobicity of the family (amphipathy profile) using a period of 3.6 residues, appropriate for α-helical structures. The glutamate transporter family contains five regions with conserved hydrophobic moments that could form amphipathic α-helices (AH1-5, Fig. 4b). AH1-4 are found in loop regions connecting the putative transmembrane helices and may therefore form membrane surface helices with the axis parallel to the plain of the membrane and the hydrophilic residues exposed to the aqueous phase. AH2 and AH4 are exclusively found in the eukaryotic transporters. The hydrophobic moment of AH5 is conserved in all members of the family and has a particularly large value of 0.45-0.6/residue in the glutamate and C4-dicarboxylate transporters, which is higher than that of the peptide melittin, a considerably amphipathic peptide of known α-helical structure (33). The hydrophobic moment is somewhat smaller in the mammalian neutral amino acid transporters and the bacterial serine transporters (0.3-0.4/residue). The putative amphipathic α-helix AH5 coincides with transmembrane segment 8 and thus provides a hydrophilic path through the membrane. It was suggested that this amphipathic membrane-spanning helix could provide part of the translocation pore (chapter 4) (147). This suggestion obtained experimental support from mutagenesis studies in GltT (chapter 6) and rat EAAT2 (14) and studies on chimeric transporters (106) (discussed below).

Within transmembrane helices the pattern of residue conservation (or substitution) can be used to discriminate between buried and lipid exposed residues (31). The amphipathic membrane-spanning helix 8 has an exceptionally large substitution moment with α-helical periodicity (0.09/residue) (chapter 4) (147). The hydrophobic face of the helix is less well conserved than the hydrophilic face and it is likely that the membrane-spanning helix has a lipid exposed hydrophobic face and a protein buried, well conserved hydrophilic face. The other putative membrane-spanning segments have considerably smaller substitution moments, but the moments of helix 1 and 3 (0.06 and 0.05 per residue, respectively) may be significant.

4. Sequence motifs

The glutamate transporter family does not contain residues that are conserved in all members. This is due to the inclusion of several only distantly related sequences in the family that have emerged from recent genome sequencing projects. For the same reason most of the previously described “signature sequences” for the glutamate transporter family are not found in all members (129). Only one sequence motive is conserved in all members of the family. This is a serine and threonine rich stretch in the reentrant loop between membrane-spanning helices 6 and 7 (52,148) (Motif A, table 2 and Fig. 1 and 4a). Serine clusters were found in the ligand binding sites of both ionotropic and metabotropic glutamate receptors and in G-protein coupled acetylcholine and biogenic amine receptors (6,113,184). It was suggested that the serine-rich stretch in the glutamate transporters might have a similar function (73,106). Mutagenesis studies in GltT confirmed that the serine-rich stretch in the reentrant loop is extremely important for the transporter’s function and may be part of the substrate-binding site (chapter 5) (148).

A second motif that was suggested to be involved in substrate binding (121) is AX(I,V,L)F(L,I)AQ (Motif C, table 2 and Fig. 1) which is located in the putative membrane-spanning helix 7 (50,147,192). The motif is conserved in the glutamate, neutral amino acid and dicarboxylate carriers, but not in most of the uncharacterized bacterial proteins. It may therefore be involved in binding of a carboxylate group of the substrate, which is the only functional group common to all substrates of the carriers that contain the motif. The motif is part of a stretch of 76 residues that is involved in the binding of dihydrokainate, a glutamate analogue that competitively inhibits glutamate transport ((173), see below). This observation is consistent with the hypothesis that the motif is involved in substrate binding.

Table 2. Sequence motifs in the glutamate transporter family

Motif	From[a]	Sequence[b]	To[a]
A	414	(ST)(STARK)S(ST)	417
B	443	PxGx(TS)xN(ML)DGxx(LI)(FY)	457
C	461	Ax(IVL)F(LI)AQ	467
D (Eukaryotic glutamate transporters)	531	DWxLDRxRTxxNVxGD	546
D (Eukaryotic neutral amino acid transporters)	531	DWxVDRxxTxxNVEGD	546
D (Bacterial glutamate transporters)	531	DRxxDMARTxxNxxG(NH)	546
D (Bacterial C4-dicarboxylate transporters)	531	DRFMSExRxxxNxxGN	546
D (Bacterial serine transporetrs)	531	GxLQDSxETALNSSTD	546

[a] Numbers refer to positions in the multiple sequence alignment of Fig. 1.
[b] When two or more residues are indicated in parentheses, either of the them is found; x refers to non-conserved residues.

The motif PXGX(T,S)XN(M,L)DGXX(L,I)(F,Y) located at the cytoplasmic interface of membrane helix 7 is present in all functionally characterized and most of the putative transporters (motif B, table 2 and Fig. 1). This motif has been subjected to extensive mutagenesis studies and is involved in cation binding (see below). The stretch that forms the amphipathic membrane helix 8 (motif D) is conserved in most members of the family, but its exact amino acid composition varies along with the substrate specificity of the transporters (table 2). The substrate specific differences in this stretch suggest that the amphipathic membrane-spanning helix 8 constitutes part of the substrate binding site or translocation pore (106,106,147). Mutagenesis studies in the glutamate transporters GltT of *B. stearothermophilus* and EAAT3 of rat confirmed that helix 8 is involved in substrate recognition (chapter 6 and (14)). Finally, the human glutamate transporter EAAT5 contains a C-terminal consensus motif for interaction with synaptic proteins that promote ion channel clustering (7,48). EAAT5 is one of the glutamate transporters that shows large substrate dependent chloride currents (see above) and clustering with components of the signal transduction pathway may point to the function of the chloride currents.

IV. Functional analysis

1. The catalytic cycle

Extensive characterization of the eukaryotic glutamate carriers EAAT1-3 has resulted in the model for the transport cycle that is shown in Fig. 6 (79). The transporters catalyze uptake, efflux and exchange of glutamate. In uptake and efflux the complete cycle is followed in forward and reverse direction, respectively, whereas during exchange steps 1 to 7 are followed alternately in forward and reverse direction. K^+ is only required to reorient the empty carrier (steps 8-10). Exchange is not dependent on potassium ions since reorientation of the empty carrier is omitted. The bacterial glutamate transporters also catalyze uptake, efflux and exchange, but none of the transport modes is believed to require potassium ions (160); the empty carrier reorients spontaneously.

The binding order of Na^+, H^+ and glutamate (steps 1-3) is a matter of debate. Kanner and coworkers (76,122) showed that exchange catalyzed by EAAT2 from rat does not require external Na^+ at saturating glutamate concentrations whereas it does at non-saturating glutamate concentrations. They concluded that all co-transported sodium ions bind to the transporter prior to glutamate binding. In contrast, Kanai *et al*. (72) found that binding of sodium ions is modulated by glutamate and in their model one Na^+ binds after the binding of glutamate (see also (30,82)). The observations indicate that the glutamate and Na^+ binding sites intimately interact which is in agreement with recent mutagenesis studies

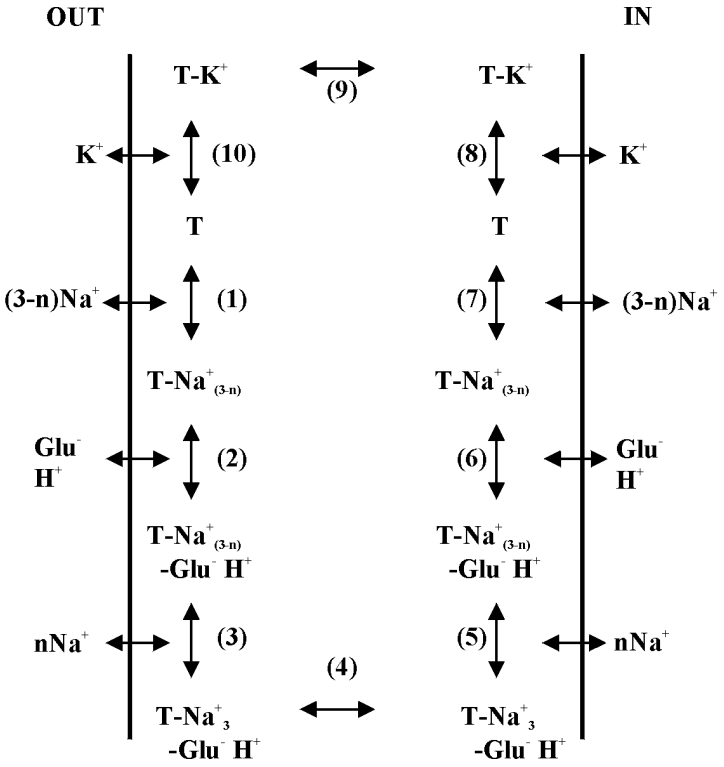

Figure 6. Schematic representation of the transport cycle of the eukaryotic glutamate transporters (79). T, transporter; glu$^-$, glutamate; n, the number of sodium ions that bind after glutamate binding. A description is given in the text.

((123,197,199), see below). Possibly the binding order depends on the applied conditions. In rat EAAT3 the cotransported proton was found to bind to the transporter prior to glutamate binding (186).

The catalytic cycle of the neutral amino acid transporters may be similar to that of the exchange mode of the glutamate transporters. The human neutral amino acid transporter ASCT1 catalyzes electroneutral exchange of amino acids that is dependent on Na^+ but not on K^+, just like exchange catalyzed by the glutamate transporters (194). A difference is the Na^+-stoichiometry since only one sodium ion was found to interact with the neutral amino acid transporters (168,194).

2. The substrate binding site

Inhibitors

Several inhibitors of the glutamate transporters have been found, some of which with different specificity for different members of the family (8,89,144,174,176). Non-competitive inhibitors include oxidizing agents like $ONOO^-$ that covalently interact with glutamate transporters and specifically inhibit the V_{max} of transport (164,165,178,179) and Zn^{2+} which is a partial inhibitor of the human and salamander glutamate transporters EAAT1 (151,175). Arachidonic acid inhibits some but stimulates other glutamate transporter subtypes (166,193).

Most of the competitive inhibitors of the glutamate transporters were found to be substrates of the proteins that are transported with a high affinity (K_m < 100 μM), e.g. D-aspartate, cysteate and *threo*-β-hydroxyaspartate (Fig. 7) (71,121,152). Only a few competitive inhibitors that are not transported have been identified such as *threo*-β-benzyloxyaspartate and kainate, a specific inhibitor of the EAAT2 subtypes (Fig. 7) (16,89,143,174). The conformations of glutamate and aspartate in the substrate binding site have been modeled by comparing the structures of transported glutamate analogues and competitive blockers of the transporters (16,17,24,89,174). It was originally proposed that glutamate binds in a folded form to the

transporters and aspartate in the extended form. In these conformations the functional groups of the transported substrates (one amino group and two carboxylate groups) can be superimposed. However, more recent data from the analysis of conformationally constrained glutamate analogues showed that compounds that mimic the folded conformation as well as compounds that mimic a stretched conformation of glutamate bind to glutamate transporters, but that only the "stretched" compounds can be transported (39,144).

The nature and diastereomeric properties of the substituents at the β-carbon (C3 position) of aspartate determine whether aspartate analogues are transported substrates or competitive blockers (89,174). Thus, derivatives of aspartate with small groups at the β-carbon, such as β-

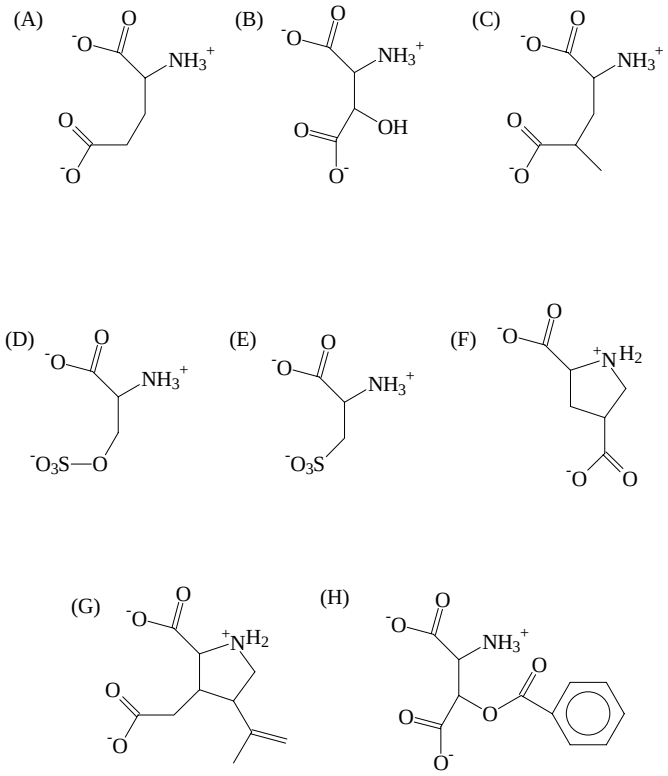

Figure 7. Structures of some transported substrates (A,B,D,E,F) and competitive inhibitors (G,H) of the glutamate transporters. 4-Methyl-glutamate (C), is a substrate of some transporters but a competitive inhibitor of others. (A), Glutamate; (B), β-hydroxy-aspartate; (D), Serine-O-sulfate; (E), Cysteate; (F), Pyrrodiline-2,4-dicarboxylate; (G) kainate; (H), β-benzyloxyaspartate.

hydroxyaspartate (Fig. 7B) and the related compounds β-acetoxyaspartate and β-propionyloxyaspartate, are transported by the bovine glutamate transporters EAAT1 and EAAT2 with high affinity (K_m = 10-100 μM). Derivatives with bulkier groups at the β-carbon, such as β-benzyloxyaspartate (Fig. 7H), are competitive blockers (K_i < 20 μM) (89,143).

The transporters are less tolerant in accepting derivatives of glutamate as substrates. (2S,4S)-4-methylglutamate (Fig. 7C) is not recognized by human EAAT1 and EAAT2, whereas its stereoisomer (2S,4R)-4-methylglutamate is a substrate for EAAT1 (K_m = 54 μM) but a competitive blocker of EAAT2 (K_i = 3.4 μM). Both stereoisomers of 4-hydroxyglutamate are transported by the human glutamate transporters EAAT1 and EAAT2, but the affinity for *erythro*-4-hydroxyglutamate is very low (K_m>1mM) (174). Substitutions at the C3 position are even less well tolerated. None of the stereoisomers of 3-methylglutamate is recognized by EAAT1, while only the stereoisomer *threo*-3-methylglutamate is bound, but not transported, by EAAT2 (K_i = 2.3 μM).

Mutagenesis

Differences in the amino acid sequences of members of the glutamate transporter family have been used as a guide in mutagenesis studies to find residues of the substrate binding site or translocation pore (22,175,199,200) (examples are listed in table 3). Mutations that completely abolish transport may be useful to delineate functionally or structurally important regions in the proteins. Thus it was shown that several conserved hydrophilic residues in α-helical membrane-spanning segment 8 (chapter 6 and (14,22,123)) are indispensable for transport activity, but the exact function of residues that are indispensable for transport function is difficult to assess. However, one mutant in membrane-spanning segment 8 of human EAAT3 (R447C) lost the ability to transport glutamate, but retained the ability to transport cysteine, a substrate of EAAT3 but not of the other glutamate transporters. It was concluded that Arg447 is involved in the binding of the γ-carboxylate group of glutamate (14). Studies on chimeras between human EAAT1 and EAAT2, which have slightly different substrate specificity (see above) and Cl^- permeability confirmed the involvement of helix 8 in substrate recognition and transport (106).

Table 3. Glutamate transporter mutants [a]

Mutated residue [b]	Protein	Characteristics	Reference
Y403 (FWC)	EAAT2 rno	cation binding affected	(197)
E404 (CDGN)	EAAT2 rno	cation binding affected	(79)
S440 (GC)	EAAT2 rno	cation selectivity and dihydrokainate binding affected	(199)
S443 (QC)	EAAT2 rno	cation selectivity affected	(199)
R447C	EAAT3 hs	glutamate binding	(14)
H146	EAAT1 hs	Zn^{2+}-binding	(175)
H156	EAAT1 hs	Zn^{2+}-binding	(175)
A395C	EAAT1 hs	glutamate binding	(139)
N206T	EAAT1 rno	glycosylation mutant	(23)
N216T	EAAT1 rno	glycosylation mutant	(23)
Y405F	EAAT1 rno	like Y403C of EAAT2 rno	(192)

[a] Only mutants affecting binding of substrates, cations or inhibitors and glycosylation mutants are listed.
[b] If a residue was replaced with multiple other amino acids, all mutants are indicated between parentheses.
[c] Only the activity of the double mutant was tested.

In rat EAAT2 mutagenesis of serine 440 in the reentrant loop between membrane-spanning helices 7 and 8 influences binding of dihydrokainate, a non-transported competitive inhibitor of EAAT2, but not of the other members of the family (199). Furthermore, analysis of chimeras between human EAAT1 and EAAT2 revealed that a stretch of 76 residues comprising the conserved motifs B and C and membrane-spanning segment 7 (Fig. 5) is also involved in the binding of the glutamate analogue dihydrokainate (173). Kanai and co-workers constructed chimeras between EAAT2 and EAAT3 from mouse and showed that a region of 38 residues comprising conserved serine-rich motif A (Fig. 5) may also contain part of the binding site for the glutamate analogue dihydrokainate (74). Dihydrokainate binds to an external site of the transporter EAAT2 (181), consistent with the presence of a reentrant loop in the region (Fig. 5). Cysteine-scanning mutagenesis of the serine rich motif A in the reentrant loop between helices 6 and 7 confirmed an important role of the motif in glutamate binding or transport (chapter 5) (148). Interestingly, in the glutamate transporter GltT the conserved serine-rich motif was shown to be accessible from both sides of the membrane. The double-sided accessibility suggests an important function in the catalytic cycle of the transporter in which the glutamate-binding site is alternately exposed to each side of the membrane. In conclusion, both reentrant loops as well as membrane-spanning segments 7 and 8 may form an important part of the substrate recognition site and translocation pore.

Protonation state of the substrate

In the human glutamate transporter EAAT3 the stoichiometry of proton: substrate symport was found to depend on the charge of the transported substrate (195). Thus transport of compounds which are anionic at physiological pH, like glutamate (pK_a = 4.5) and cysteate (pK_a = 1.5; Fig. 7E), is associated with the symport of one proton. However, transport of cysteine, a low affinity substrate for EAAT3 that is neutral at physiological pH (pK_a = 8.3), is transported without a proton. Based on these observations it was suggested that the anionic substrates are transported in the protonated state, i.e. as glutamic acid or cysteic acid (195). This mechanism of proton transport is feasible if the pK_a of the anionic substrates is raised sufficiently in the substrate-binding site of the transporter. This can be achieved by destabilizing the negative charge on the substrate in an apolar or negatively charged surrounding. However, this mechanism may be improbable since some substrates, like serine-O-sulfate (pK_a<0; Fig. 7D), are so acidic that protonation is not likely to occur (176). Alternatively, the observed substrate dependent proton stoichiometry could be explained if the formation of a hydrogen bridge between the substrate and a residue in the substrate-binding site would be essential. Then, transport of anionic substrates requires a proton to bind in the substrate-binding site to form the hydrogen bridge whereas uncharged substrates, like cysteine and glutamic acid, provide the proton for the hydrogen bridge themselves. This mechanism implies that the substrate-binding site can exist in a protonated and an unprotonated state. The symported proton is shared by the substrate and the proton-accepting residue in the substrate-binding site.

Only at low pH glutamate and cysteate are also transported by the neutral amino acid transporters ASCT2 from mouse, rabbit and human (81,154,168). The pH dependence of transport of the anionic compounds is caused by protonation of a residue on the transporter, possibly a histidine (154). Glutamate may therefore be transported by ASCT2 in a similar way as by the glutamate transporters (168). However, unlike the glutamate transporters that may have a protonated substrate binding site at physiological pH the neutral amino acid transporters are optimized for transport of neutral compounds and are normally not protonated. Nevertheless, since glutamate is a substrate for both the glutamate transporters and the neutral amino acid transporters the substrate binding pockets of the transporters may have a similar spatial structure (168). A further indication for this suggestion is the observation that cysteine, a common substrate for the neutral amino acid transporters, is transported with low affinity by the glutamate transporter EAAT3 from human (K_m = 190 μM) (196).

3. The cation binding sites

Extensive mutagenesis studies in the conserved motif B at the cytoplasmic end of membrane-spanning helix 7 in EAAT2 from rat have shown that two residues (Y403 and E404) are involved in the binding of potassium ions (79,197). Transporters in which these residues are conservatively mutated are unable to complete the transport cycle shown in Fig. 6 because they cannot reorient the empty carrier, which is due to a defect in K^+-binding and/or translocation. These mutant transporters are however able to catalyze exchange that is K^+-independent. Cysteine modification and protection studies on the Y403C mutant showed that this residue is alternately accessible from either side of the membrane depending on the presence of substrates or transport blockers (192). This is in agreement with the alternating accessibility of the K^+-binding site from both sides of the membrane in the transport cycle (Fig. 6). Interestingly, E404 in EAAT2 of rat is conserved in all eukaryotic glutamate transporters but not in the bacterial carriers and the neutral amino acid transporters (Fig. 1). This correlates with the observations that the neutral amino acid transporters are K^+-independent obligate exchangers and that the bacterial transporters are glutamate-cation

symporters. Y403 of rat EAAT2 is not conserved in the neutral amino acid transporters either, but it is in the bacterial carriers.
Several mutagenesis studies in rat EAAT2 indicate that the binding sites for glutamate, K^+ and Na^+ intimately interact. Mutations of Y403 and E404 that affect interaction with K^+ also affect the Na^+ and glutamate selectivity of the transporters, respectively, (123,197). The mutant E404D strongly prefers aspartate over glutamate, whereas the mutants Y403W and Y403F have an increased affinity for sodium ions but lost the strict dependency on Na^+ and also accept lithium and cesium ions. The proximate residues 396 to 400 in EAAT2 of rat that are indispensable for transport were suggested to be part of the Na^+-binding site(s) (192). A serine residue (S440) in the reentrant loop between helices 7 and 8, which is involved in binding of the glutamate analogue dihydrokainate (see above), is also critical for sodium ion selectivity. Mutant S440G enables not only sodium ions but also lithium ions to drive the uptake of glutamate (199). In this respect the mutant behaves similarly as mutants Y403W and Y403F and the two residues may be in close proximity in the three dimensional structure.

V. Prospects

As long as a high resolution structure of (one of) the members of the glutamate transporter family is not available, structural features have to be obtained from biochemical and biophysical experiments. Mutagenesis studies and studies on chimeric transporters have so far proved to be very informative in obtaining information on the function of individual residues and domains, respectively. Mutagenesis is likely to remain useful as long as mutants with interesting properties can be found. One way to find interesting residues is to look for second site revertants of mutant transporters that are inactive. For this the bacterial members of the family can be used.
Cysteine scanning mutagenesis is likely to remain a major source of structural information. This technique has been extensively used in the study of the lactose transporter LacY from *E.coli* (45,69) and the first results from this technique are now available for members of the glutamate transporter family. To fully benefit from the possibilities offered by cysteine scanning mutagenesis it will be necessary to use purified and reconstituted transporters. Such a pure system allows the use of a scala of spectroscopic techniques to study both structural and dynamic properties of the transporters. The glutamate transporters EAAT2 of rat and GltT of *B. stearothermophilus* have been purified to homogeneity and reconstituted into proteoliposomes (chapter 3) (26,47). The bacterial system is suitable for the expression and large scale purification of both wild-type and mutant transporters (chapter 5 and 6).

Outline of this thesis

The structure of the glutamate transporters was studied by bioinformatical and experimental approaches. In chapter 2 a bioinformatical method is described to classify membrane protein structures on the basis of their hydropathy profiles. The analysis indicates that the glutamate transporter family forms a unique structural class of membrane proteins, which is different from any other family of secondary transporters. In chapter 4 the members of the glutamate transporter family were examined for patterns of residue hydrophobicity and residue conservation (or substitution) in their amino acid sequences and the results indicate the presence of an unusually amphipathic membrane-spanning α-helix in the glutamate transporters.

Experimental studies to elucidate structural features of the glutamate transporter GltT of *Bacillus stearothermophilus* are described in chapters 3-6. The basic methods for the experimental approach are presented in chapter 3. GltT was tagged with an N-terminal His-tag, expressed in *E. coli* and the His-tagged protein was purified and reconstituted into proteoliposomes. The expression procedure was subsequently used for the studies described chapters 4-6, whereas the purification and reconstitution procedures were used again in the experiments presented in chapters 5 and 6.

In chapters 4-6, the *phoA*-gene fusion method and cysteine-scanning mutagenesis were applied to study the structure and function of the C-terminal half of the glutamate transporter GltT. A reentrant loop and an amphipathic membrane-spanning α-helix were found. In chapter 7 the results are discussed together with recent results from related transporters and a model for the membrane topology is described. Furthermore, hypotheses on the functional importance of the unconventional structure of the glutamate transporters are given.